(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 May 2001 (25.05.2001)

PCT

(10) International Publication Number
WO 01/36449 A1

(51) International Patent Classification[7]: C07K 1/26, B01D 61/46

(21) International Application Number: PCT/AU00/01391

(22) International Filing Date:
14 November 2000 (14.11.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PQ 4058 15 November 1999 (15.11.1999) AU
PQ 8727 12 July 2000 (12.07.2000) AU

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published:**
— *With international search report.*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 01/36449 A1

(54) Title: MULTI-COMPARTMENT ELECTROPHORESIS

(57) Abstract: A novel multi-compartment electrolyser design is used to prefractionate complex protein mixtures, for use prior to the implementation of 2-D maps. Such sub-fractionation processes can effectively remove, via suitable narrow range isoelectric membranes, proteins present in large excess in a cell lysate or in body fluids. In turn, the remaining protein mixture, devoid of such major components, can be loaded in a 2-D map at much higher levels, thus ensuring a greater sensitivity and detection capability of low-abundance proteins. The apparatus is a multi-compartment electrolyser, which is characterised in that recirculation of the samples is not required. The apparatus produces protein fractions which are fully compatible with the subsequent 2-D protocols, since it is based on a focusing technique, which yields samples highly concentrated and devoid of salts and buffers.

# MULTI-COMPARTMENT ELECTROPHORESIS

## Field of the Invention

The present invention relates to an apparatus and a method utilising that apparatus for sub-fractionation and subsequent separation of the fractions from highly complex protein/peptide mixtures, such as those found in total cell lysates, body fluids (e.g., plasma, sera, cerebrospinal fluid, urine) and tissue extracts in general.

## Background of the Invention

For the last 25 years, two-dimensional polyacrylamide gel electrophesis, (2-D PAGE) has been the technique of choice for analysing the protein composition of a given cell type and for monitoring changes in gene activity through the quantitative and qualitative analysis of the thousands of proteins that orchestrate various cellular functions. Notwithstanding its extraordinary resolving power, even 2-D PAGE appears to have reached a plateau in relation to the number of proteins which can be resolved and detected in a single 2-D map. The advent of immobilized pH gradients (IPG) has enabled an improvement in resolution, however the resolving power cannot be further improved. Solubilizing cocktails have improved, due to the introduction of new surfactants and new reducing agents, although, progress seems now to be levelling off (T. Rabilloud, C. Adessi, A. Giraudel, J. Lunardi, Electrophoresis 18, 1997, 307-316) (M. Chevallet, V. Santoni, A. Poinas, D. Rouquie, A. Fuchs, S. Kieffer, M. Rossignol, J. Lunardi, J. Garin, T. Rabilloud, Electrophoresis 19, 1998, 1901-1909) (B.R. Herbert, M.P. Molloy, A.A. Gooley, B.J. Walsh, W.G. Bryson and K.L. Williams, Electrophoresis, 19, 1998, 845-851). In relation to staining protocols no increment in detection sensitivity in the last five years appears to have occurred. This includes fluorescent stains such as Sypro Ruby, which is claimed to be as sensitive as a good silver stain, which have detection limits around 1-2ng. Yet, the enormous complexity of the proteome (it is hypothesised that up to 100,000 genes could be present in the human genome, but the proteins in the proteome could be as many as 1 million, if one considers all the possible post-translational modifications) calls for improvements in resolving power, possibly coupled to increments in detection sensitivity for tracking trace components.

There remain two main problems in 2-D map analysis: one is the concentration, below a minimum critical detection level, of a number of low-abundance polypeptides expressed by the cells; the other is the removal of over abundant proteins which mask minor proteins present in the 2-D map. A case in point is represented by the 2-D analysis of human plasma. Typically, a few thousand polypeptide chains are separated and revealed in a standard 2-D map, however, this number is thought to be a significant underestimate of the total number of expressed polypeptides. One way to overcome the problem would be to massively overload the sample in the first dimension, isoelectric focusing (IEF) gel, so as to augment the load of the least-abundant proteins. This is not possible, since, due to the overloading of albumin (which, alone, represents ca. 60% of the total proteins present in human sera), severe smears and precipitation occur across most of the pH gradient, greatly distorting and obscuring the pattern. Even if it were possible to selectively remove albumin (which at present has not been properly achieved) there remains the problem that, for appropriate resolution, one would have to resort to rather narrow pH gradients (1 pH unit wide or less) in pI regions very rich in polypeptides (the pH 4-6 region contains 60% of all expressed polypeptides). When narrow pH gradients, though, are loaded with an entire cell lysate (which contains all extractable proteins expressed by a given cell type, typically having pI values encompassing the pH 3-11 scale), massive precipitation ensues of all proteins non-isoelectric in such narrow pH intervals. These precipitates also trap proteins which have isoelectric points in the given pH interval; additionally, they greatly disturb the focusing process.

The present invention seeks to provide a new instrument which is capable of being operated to provide improvements in resolving power and preferably improvements in detection sensitivity.

## Summary of the Invention

In a first broad aspect, it is the underlying idea of the present invention to use a novel multi-compartment electrolyser design to pre-fractionate complex protein mixtures, for use prior to the implementation of 2-D maps. Such a sub-fractionation processes can effectively remove, via suitable narrow range isoelectric membranes, proteins present in large excess in a cell lysate or in body fluids. In turn, the remaining protein mixture, devoid of

such major components, can be loaded in a narrow pH range 2-D map at much higher levels, thus ensuring a greater sensitivity and detection capability of low-abundance proteins. The apparatus embodying the present invention produces protein fractions which are fully compatible with the subsequent 2-D protocols, since it is based on a focusing technique, which yields samples highly concentrated and devoid of salts and buffers.

The process of the present invention may be carried out using a multi-compartment electrolyser, which is characterised in that recirculation of the samples is not required.

Thus, in one aspect, the present invention provides a process for electrophoretic separation of a mixture of macromolecules into groups comprising the steps of placing a sample mixture of macromolecules in a separation apparatus comprising a series of chambers separated by isoelectric membranes of known pI, the apparatus also having chambers located at each end of the series of chambers containing means for applying an electric field across the series of chambers; and

applying an electric field across the chambers to separate the macromolecules on the basis of their isoelectric point characterised in that the sample mixture is agitated within the chambers and is not recirculated

Each chamber preferably defines a well or recess adapted to receive a magnetic stirrer bar.

Electrodecantation may be prevented by placing the device in a multi-place magnetic stirring platform and mixing the contents of each chamber of the electrolyser with the magnetic stirrer bar.

The separator apparatus of the present invention may utilise an associated platform which provides power, cooling and a magnetic stirrer platform. Clearly, the location of the magnets in the magnet array of the magnetic stirrer platform have to coincide with the location of the stirrer magnets in the electrolyser. Heat dissipation is implemented by the use of embedded Peltier elements in the magnetic stirrer platform which can be regulated to maintain the correct temperature.

The platform may also be configured to accommodate a plurality of gel trays for isoelectric focusing in carrier ampholyte pH gradients or immobilised pH gradients.

Typically, in the first operational mode (pre-fractionation) the multi-compartment electrolysers are assembled from a plurality of separate

chambers, operating in an electric field, with a set of isoelectric membranes (having pI values increasing monotonically from anode to cathode) able to trap a desired protein population within a given chamber. In the pre-fractionation mode, using one or more multi-compartment electrolysers, the device can be operated under denaturing conditions (with mixtures of chaotropes, surfactants, organic solvents and cysteine-reducing and/or cysteine-alkylating agents added to both sample and relevant chamber solutions), as customarily done in 2-D map analysis, in which case the temperature will be set to about 20°C. Alternatively, the device can be operated under native conditions, in the absence of denaturants, when native proteins are required for further analysis exploiting biological activity and this it typically done at about 4°C.

The isoelectric membranes may be made either with standard acrylamide monomers or with stable acrylamide derivatives, such as N-acryloyl amino ethoxy ethanol or N-acryloyl amino propanol, or mixtures thereof. Membrane pH is defined by a suitable choice of buffering and counter-ion species, which are acrylamido-derivatives able to be copolymerised with polyacrylamide, or its derivatives, and possessing good buffering power.

The isoelectric membranes may be made macroporous either by high levels of cross linkers, or by lateral aggregation of the acrylamide chains or by a combination of both means.

The sub-fractionation of complex protein/samples may be obtained under native conditions in presence of suitable solubilizers compatible with maintenance of protein integrity, such as sugars, non-detergent sulfobetaines, glycerol, ethylene and propylene glycols and mixtures thereof.

The sub-fractionation of complex protein/peptide mixtures may be obtained under denaturing conditions in presence of appropriate solubilizing cocktails, such as organic solvents, chaotropes, neutral and zwitterionic surfactants and amido-sulfobetaines and, when required, in presence of suitable disulfide bridge reducing and/or alkylating agents.

The preferred embodiment for sample application may be by pulse-loading in a single chamber of the electrolyser, said chamber being preferably in the neutral or basic region of the pH scale.

The sub-fractionation process may be performed in a cascade fashion, such that a wider pH fraction obtained in a first run in the electrolyser, and

may subsequently be further fractionated in a very narrow pH window, as required by the sample complexity.

As discussed above, the device described in the present invention also comprises a second operational mode for simultaneous separation of certain protein fractions, using isoelectric focusing in carrier ampholyte pH gradients or immobilised pH gradients. To simplify the interface between the fractionation mode and the isoelectric focusing mode it is possible to invert a multi-compartment electrolyser and directly load the fraction solutions into a tray for isoelectric focusing.

## Brief Description of the Drawings

Specific embodiments of the invention will now be described by way of example only and with reference to the accompanying drawings in which:-

Figure 1 is a schematic exploded view of a multi-compartment electrolyser apparatus embodying the present invention;

Figure 1a illustrates a suspended magnetic stirrer element;

Figure 2 is a schematic view of two multi-compartment electrolysers set up accommodated on an electrophoresis platform containing a power supply, Peltier cooling and a multi-place magnetic stirrer;

Figure 3 is a schematic view of two trays for isoelectric focusing accommodated on an electrophoresis platform containing a power supply; Peltier cooling and a multi-place magnetic stirrer;

Figure 4 is a silver stained 2-D map of a pI 4-5 fraction of *E. coli*, as purified in the multi-compartment electrolyser of Fig. 1, run in a 7cm pH 3-10 IPG strip in the first dimension; and

Figures 5a and 5b are 2-D maps of a plasma sample in a narrow pH 3-6, first dimension Immobiline gel, with Figure 5a showing unfractionated plasma and Figure 5b showing pre-fractionated plasma.

## Description of the Preferred Embodiments of the Invention and Examples

Referring to the drawings, Figure 1 shows a disassembled separation apparatus in the from of a multi-compartment electrolyser apparatus 10. The apparatus includes five chamber blocks, defining three inner fractionation chambers 12 and two, outer, electrode chambers 14. Each chamber block is generally square in transverse cross-section. A cylindrical through bore 16 extends through the centre of each of the fractionation chamber blocks and

part way into the outer electrode chambers 14. Four narrower through bores 18 located at the corners of the square cross-section of the blocks extend through all five chambers. The bores 18 receive threaded tie rods (not shown) which can be used to align the bores together and join the chambers together. Each chamber also includes a sample inlet 26 in the upper face of the chamber block which extends from the upper face of the block down to the bore 18.

Each chamber 12 defines a shallow well or recess 20 at the base of the bore which is typically co-axial with the sample inlet. In use, the well receives a magnetic stirrer. not illustrated.

One side of each chamber block 12 defines an annular spigot 22 extending around the bore 16. The opposite side defines a corresponding recess 24. The open side of one electrode chamber 14 defines a recess. The open side of the other electrode chamber 14 defines a spigot.

The multi-compartment electrolyser apparatus is assembled by placing septa or dividing walls (not shown) between adjacent chambers and inserting tie rods through the holes in each chamber and tightening until the unit is sealed. The septa between the various chambers are isoelectric. buffering membranes, cast onto a support of glass fibres or other suitable material. The membranes are sandwiched between two washers and an O-ring is disposed outside the membrane to provide additional sealing. Such membranes are flow–tight and ensure proper pH control.

Figure 1a illustrates a cap 30 for closing the sample inlets 26. A magnetic stirrer 32 (typically a small teflon coated bar magnet) is suspended from the cap 30 on a wire 33, thread or the like via a swivel joint 34. The length of the wire is such that the stirrer located in the well 20.

The novel apparatus of the present invention may be used for sub-fractionation of entire cell lysates, tissue extracts, body fluids and of any complex protein/peptide mixture, as a sample treatment, prior to a subsequent two-dimensional analysis step.

The membranes at each end of a chambers have pH values encompassing the pIs of the proteins to be confined within said chamber. The electrode solutions and sample solutions are added and removed via the sample inlets 26 in the top of each chamber. The sample inlets also allow excess fluid in a particular chamber to escape.

Although the device shown in Fig.1 is composed of five chambers, two of which are the electrode chambers at the ends, and three sample chambers, it will be clear to those skilled in the art that a multi-compartment electrolyser of this design could be made to contain more or less than five chambers.

The chamber blocks may be made of machined acrylic but will more typically be moulded in a disposable plastic material. The problems of sample carry over from one separation to another would require non-disposable chambers to be scrupulously cleaned after each use and it is more efficient in terms of resources because of the amount of cleaning necessary to simply throw the chamber blocks away after use. The chambers will typically have a volume of 80ml. although smaller volumes, say 5ml could be used.

The chamber does not require recirculation of the samples as is the case with existing multi-compartment electrolysers. Instead, the magnetic stirrer moves the sample liquid around the chamber and ensures that the proteins and the like in the sample move past the membranes and also prevents electrodecantion. This also prevents losses of protein through recirculation, obviates the need for a peristaltic pump and reduces the number of seals in the apparatus. The manner of mounting the magnetic stirrers in the chamber suspended from the cap means that the stirrers can be easily removed from the chamber for re-use. whilst the chamber can be disposed of.

Figure 2 illustrates the use of a plurality of multi-compartment electrolysers 10, as shown in Fig.1, on an electrophoresis platform 50 with an integral power supply, multi-position magnetic stirrer and Peltier cooling. (The floor 52 of the platform is cooled by the Peltier elements) Although Figure 2 shows the use of two multi-compartment electrolysers 10 on a Peltier cooled multi-position magnetic stirrer platform. it will be clear to those skilled in the art that an electrophoresis platform of this design could be made to contain more or less than 2 multi-compartment electrolysers.

Figure 3 illustrates using the same electrophoresis platform 50 as shown in Fig. 2 to accommodate a plurality of trays 54 as described in applicant's co-pending PCT patent application No PCT/AU/01065 (the contents of which are incorporated herein by reference). The trays are specially designed for isoelectric focusing in carrier ampholyte or

immobilised pH gradient gels. Again the platform could be made to contain more or less than 2 isoelectric focusing trays.

As an example of the utility of the present invention for pre-fractionation of complex mixtures, Figure 4 shows a pI 4.0-5.0 fraction fractionated in the multi-compartment electrolyser shown in Fig. 1. A IPG 3-10 strip was utilised for the first, IEF dimension, thus demonstrating that indeed, in the 2-D map, only this acidic fraction could be displayed, all other proteins in the remainder of the pH scale having been efficiently removed. Figure 5 shows 2 silver stained pH 3-6 IPGs with whole plasma in the left panel and fractionated plasma in the right panel. The 3 black arrows show matching areas on the 2 gels. The vertical arrow on the right panel indicates pH 5.6 which was the pH of the delimiting membrane between the acidic and albumin chambers. It is clear that the fractionation has removed albumin, which is the most abundant protein on the whole plasma gel (left panel). In addition, the 3 abundant spots at the pH 3 end of the whole plasma gel are not present in the fractionated gel, which indicates that they may be precipitated albumin. These spots are at the same Mr as albumin on the whole plasma gel and are connected by a continuous horizontal streak to the albumin at the pH 6 end of the gel.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

CLAIMS

1. A process for electrophoretic separation of a mixture of macromolecules into groups comprising the steps of placing a sample mixture of macromolecules in a separation apparatus comprising a series of chambers separated by isoelectric membranes of known pH, the apparatus also having chambers located at each end of the series of chambers containing means for applying an electric field across the series of chambers; and

applying an electric field across the chambers to separate the macromolecules on the basis of their isoelectric point characterised in that the sample mixture is agitated within the chambers and is not recirculated.

2. A process as claimed in claim 1 wherein each chamber defines a well or recess adapted to receive a magnetic stirrer bar.

3. A process as claimed in claim 2 wherein the separation apparatus is placed on a magnetic stirring platform during the separation process and the contents of each chamber of the electrolyser are mixed by turning magnetic stirrer bars located in the wells.

4. A process as claimed in any one of claims 2 to 3 wherein the separation apparatus is located on an associated platform which provides power, cooling and a magnetic stirrer platform.

5. A process as claimed in claim 4 wherein the platform is configured to accommodate a plurality of gel trays for isoelectric focusing in carrier ampholyte pH gradients or immobilised pH gradients.

6. A process as claimed in any one of the preceding claims wherein the separation apparatus comprises a plurality of separate chambers with a set of isoelectric membranes having pI values increasing monotonically from anode to cathode able to trap a desired protein population within a given chamber.

7. A process as claimed in claim 6 wherein the process is carried out under denaturing conditions.

8. A process as claimed in claim 6 wherein the process is carried out under native conditions, in the absence of denaturants.

9. A process as claimed in any one of claims 6 to 9 wherein the isoelectric membranes are made with standard acrylamide monomers or with stable acrylamide derivatives, such as N-acryloyl amino ethoxy ethanol or N-acryloyl amino propanol, or mixtures thereof.

10. A process as claimed in claim 9 wherein the isoelectric membranes may be made macroporous either by high levels of cross linkers. or by lateral aggregation of acrylamide chains or by a combination of both means.

11. A process as claimed in any preceding claim 9 wherein the sample is loaded into a single chamber of the separation apparatus said chamber being preferably in a neutral or basic region of the pH scale.

12. An apparatus for separating a mixture of components using isoelectric focussing in a liquid media comprising a series of chambers separated by isoelectric membranes of known pH, the apparatus also having end chambers located at each end of the series of chambers including means for applying an electric field across the series of chambers characterised in that a means for agitating the liquid media within the chambers without recirculation of the liquid is provided in the chambers.

13. An apparatus as claimed in claim 12 wherein a means for agitating is provided in each chamber apart from the end chambers.

14. An apparatus as claimed in claim 12 wherein a means for agitating is provided in each chamber including the end chambers

15. An apparatus as claimed in any one of claims 12 to 14 wherein the means for agitating includes a well or recess adapted to receive a magnetic stirrer bar.

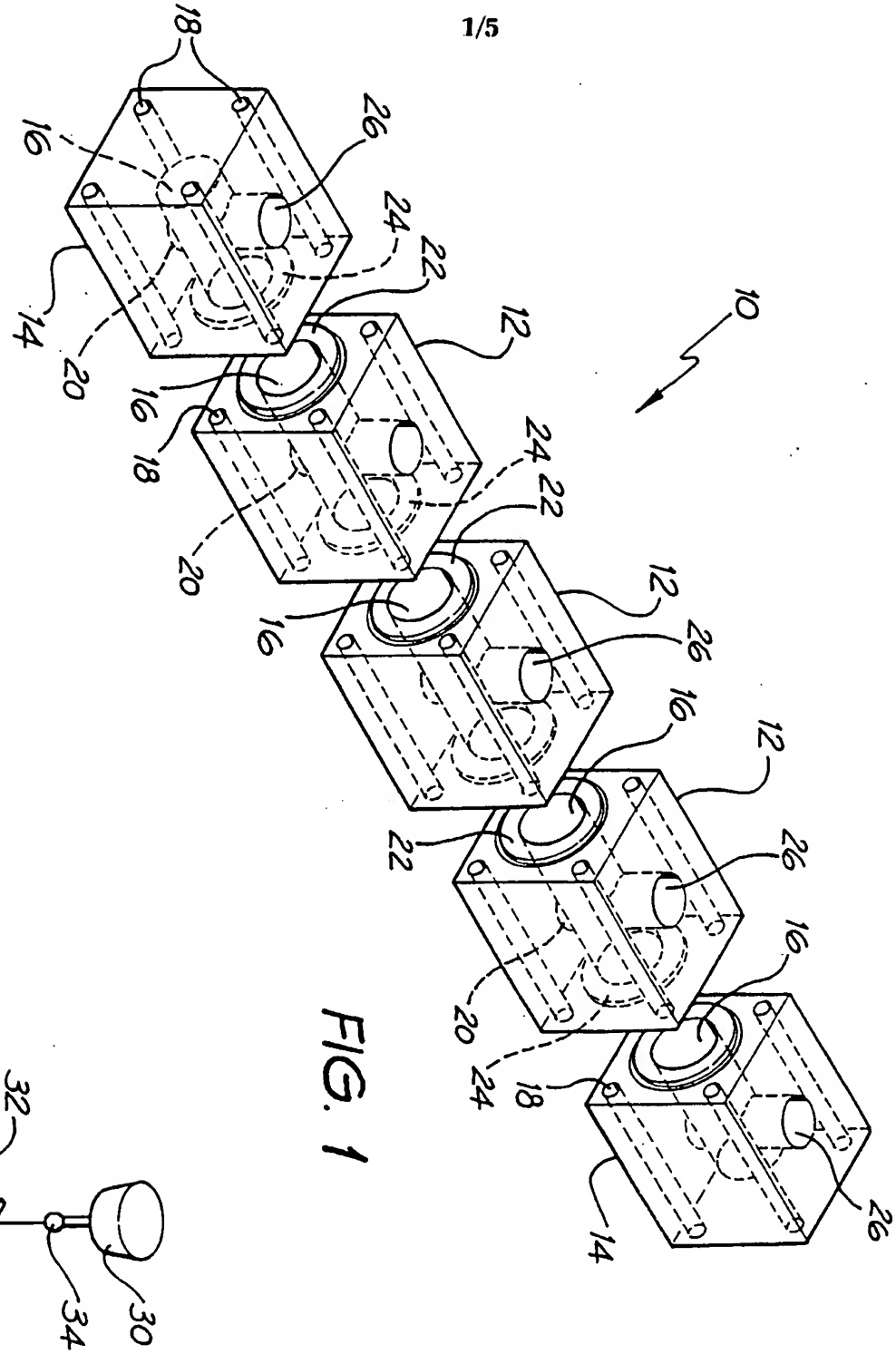
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FIG. 1


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FIG. 1a

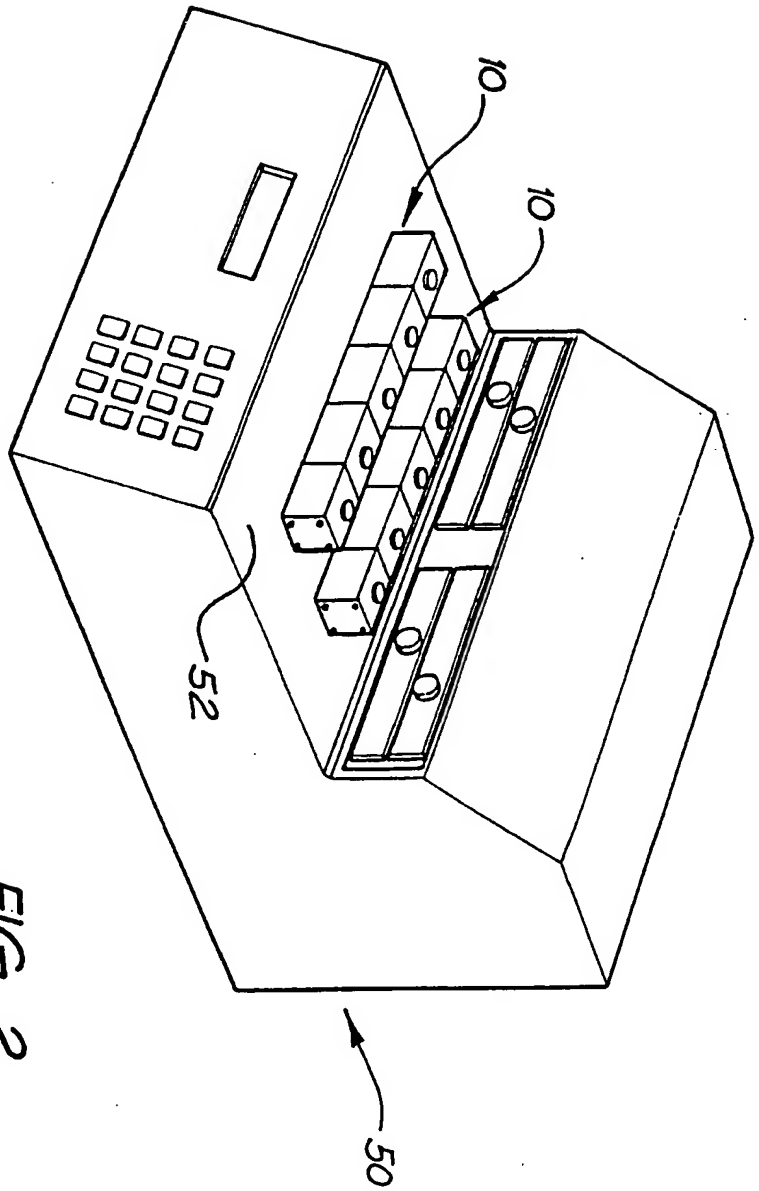
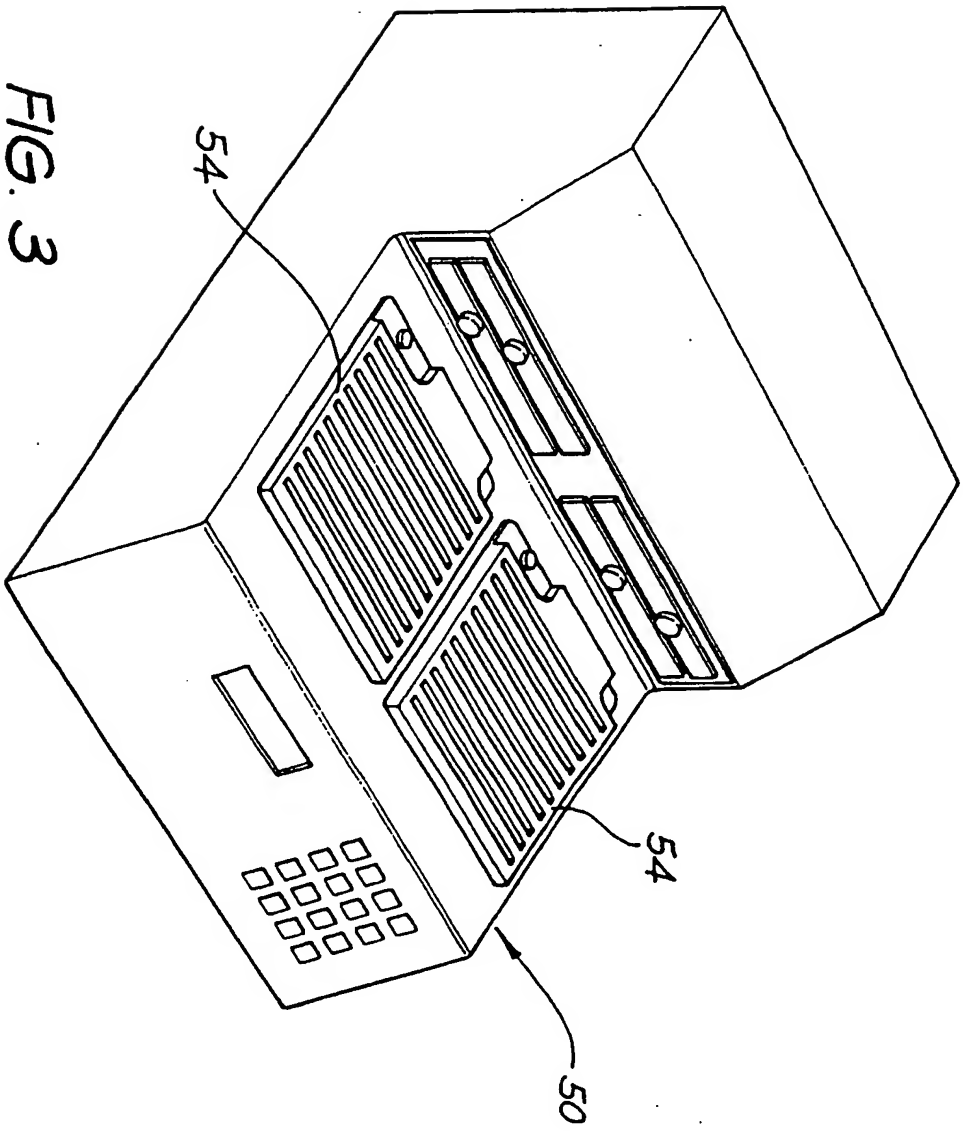
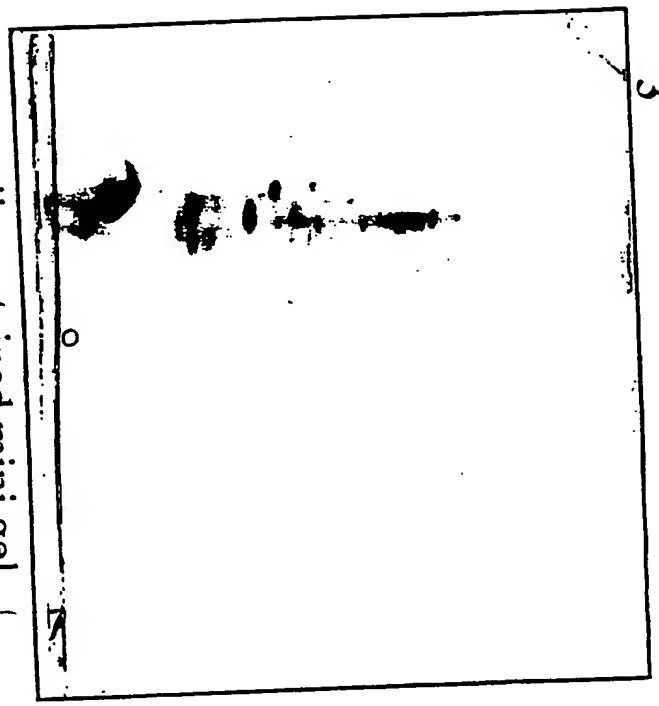



FIG. 2

FIG. 3






*E. coli* pH 4-5 fraction

FIG. 4

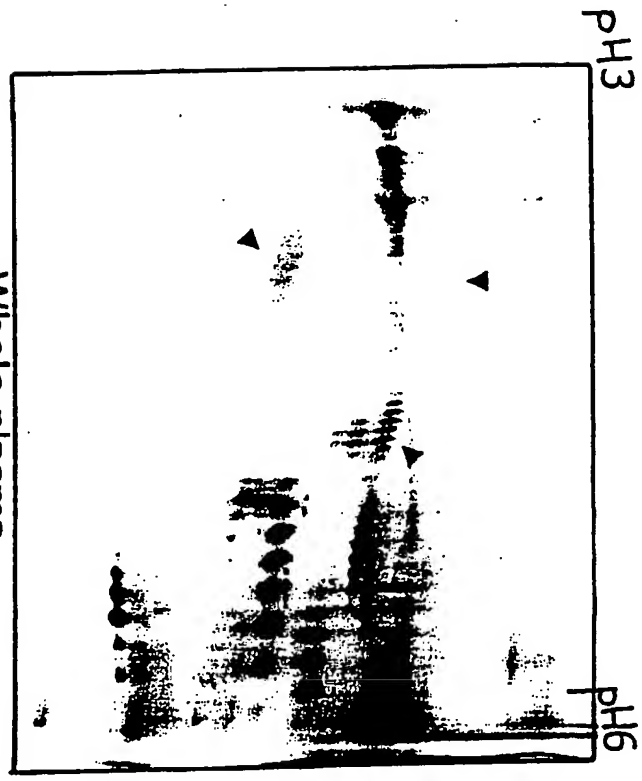



FIG.5a

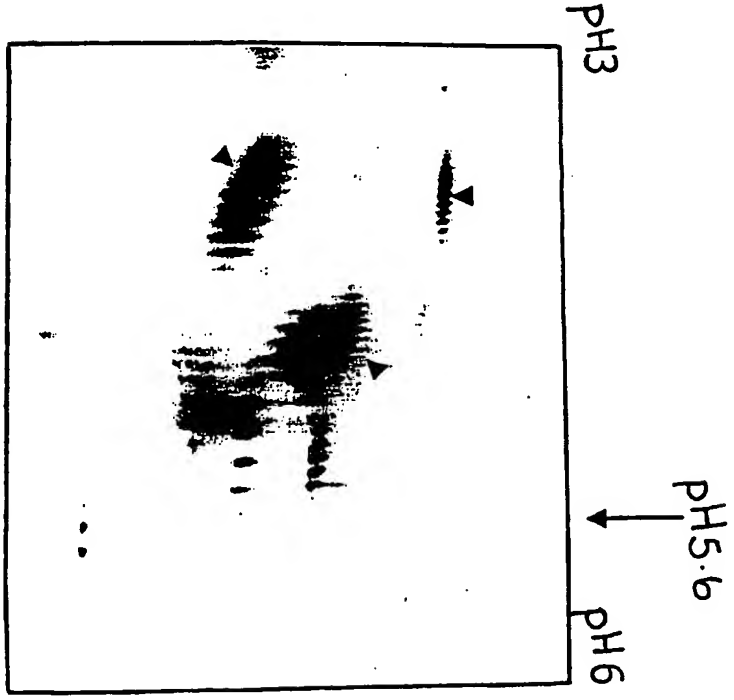



FIG.5b

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/01391

**A. CLASSIFICATION OF SUBJECT MATTER**

Int. Cl. [7]: C07K 1/26; B01D 61/46

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN: Files: CA, MEDLINE; Keywords: electrophor?, system, apparatus, preparative

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | RIGHETTI PG et al *Preparative Protein Purification in a Multi-Compartment Electrolyser with Immobiline Membranes* Journal of Chromatography, 475 (1989) 293-309 See Abstract, pages 296-298 with reference to Figures 1-4. | 1-15 |
| X | WO, A, 9200795(SERVAFEINBIOCHEMICA GmbH & CO) 23 January 1992. See Figures, claims 8 and 9. | 1-15 |
| A | US, A, 3533935 (STEERE RL, et al) 13 October 1970. See Figures 4 and 5. Column 5 lines 8-24. | 1-15 |

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Date of the actual completion of the international search
22 January 2001

Date of mailing of the international search report
13 February 2001

Name and mailing address of the ISA/AU

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INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU00/01391

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent Document Cited in Search Report | | Patent Family Member | | | | | |
|---|---|---|---|---|---|---|---|
| WO | 92/00795 | DE | 4021728 | EP | 539399 | JP | 5508577 |

END OF ANNEX